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A new polyvalent melanoma cell vaccine (MCV) was administered to 136 stage IIIA and IV (American Joint Committee on Cancer) melanoma patients. Induction of cell-mediated and humoral immune responses to common melanoma-associated antigens present on autologous melanoma cells was observed in patients receiving the new MCV. This was accompanied by increased activation of tumor-infiltrating lymphocytes. Survival correlated significantly with delayed cutaneous hypersensitivity ($p = 0.0066$) and antibody responses to MCV ($p = 0.0117$). Of 40 patients with evaluable disease, nine (23%) had regressions (three complete). From our historical database of 126 stage IIIA and 1275 stage IV melanoma patients, there were no significant changes in the natural history of metastatic melanoma during the past 20 years. Univariate and multivariate analyses demonstrated prognostic significance for site of metastases ($p = 0.0001$) and immunotherapy with the new MCV ($p = 0.0001$). Overall our new MCV increased the median and 5-year survival of stage IIIA melanoma patients with regional soft tissue metastases twofold ($p = 0.00024$), and stage IV patients threefold ($p = 0.0001$) compared with previous immunotherapy and other treatments.

ONCE MELANOMA HAS metastasized to distant sites, prognosis is guarded, with most series showing an overall median survival varying between 4 and 6 months.[1,2] Long-term survival is extremely rare. In a series from the Mayo Clinic reviewed by Ahmann et al.,[3] there were only ten 5-year survivors (2%) among 502 patients with advanced melanoma treated with chemotherapy. Our recent review of the John Wayne Cancer Institute (JWCI) database for American Joint Committee on Cancer (AJCC) stage IV metastatic disease found that the median survival (7.5 months) of our patients was slightly longer than that reported by most centers. Also, long-term survival appeared enhanced, with 6% of patients surviving at least 5 years, from a large series of 1275 patients treated by the staff of JWCI over the past 20 years. This paper presents the results of a phase II study with active specific immunotherapy using a new polyvalent melanoma vaccine (MCV) in advanced stage metastatic melanoma and compares survival of these immunotherapy patients with that of patients from our historical database.

The primary goal of our research during the past 25 years has been to develop more effective methods for the active specific immunotherapy of melanoma. The conceptual basis for our focus has been our original observation that the intratumoral injection of cutaneous metastases in melanoma patients with bacillus Calmette-Guérin (BCG) resulted in systemic enhancement of active immunity, producing rising titers of anti-melanoma antibodies and regression of other uninjected metastatic cutaneous lesions.[4-6] Biopsy of uninjected melanoma lesions that showed clinical regression demonstrated intense lymphocytic infiltration.

We experienced limited success with our initial attempts to reproduce these observations by active immunotherapy with the intradermal or intralymphatic injection of a randomly selected tumor cell vaccine of unknown antigenicity, which was composed of irradiated allogeneic mel-

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regression, patients were treated by excision or hyperthermic perfusion, as previously described.[6,24,25] Those with metastases to visceral sites were usually managed with chemotherapy, but some patients underwent surgical resection of metastases and immunotherapy with BCG by the tine technique[6,26] or with a previously developed melanoma cell vaccine.[7] Patients whose disease progressed while on immunotherapy were treated with systemic chemotherapy consisting of single agents, such as Dacarbazine, or one of the combination regimens, such as BOLD (Bleomycin, vincristine, CCNU and DTIC),[27,28] or the newer cisplatin-based regimen (CDDP, DTIC, BCNU, and Tamoxifen).[29]

Active Specific Immunotherapy

Patients. Patients with regional (AJCC stage IIIA) or remote soft tissue or visceral metastases (AJCC stage IV) were eligible. Patients were either NED after excisional biopsy or resection of their metastatic lesions or had objectively measurable disease (AWD) at the start of therapy. Patients with prior immunotherapy, chemotherapy, or radiation therapy were deemed ineligible until 30 days after the last therapy. Patients with brain metastases were not considered unless their metastases had been resected, or brain radiation had been completed, and they were off immunosuppressive steroid medications for treatment of brain edema for at least 30 days.

TABLE 2. *Distribution of Prognostic Factors Among Patients With AJCC Stage IV Melanoma Who Were Receiving Vaccine Compared With Those Who Were Receiving Other Therapy*

Variable	N	Melanoma Cell Vaccine	N	Other Therapy
Sex				
M	43	57%	761	53%
F	32	43%	442	37%
Age (yr)				
<30	13	24%	162	16%
30–50	24	44%	407	41%
50+	18	32%	423	43%
Depth of primary lesion (mm)				
<1.5	14	31%	199	34%
>1.5	32	69%	380	66%
Primary site				
Extremity	19	25%	300	25%
Nonextremity	56	75%	903	75%
No. of metastatic sites				
1	49	65%	781	65%
2	13	17%	245	20%
≥3	13	17%	177	15%
Site of first metastases				
Lung	27	36%	439	36%
Skin, nodes	29	39%	256	21%
Brain	5	7%	193	16%
Liver	5	7%	168	14%
Bone	3	4%	80	7%
Gastrointestinal tract	6	8%	57	5%
Other	0	0%	10	1%

TABLE 3. *Distribution of Prognostic Factors Among Patients With AJCC Stage IV Melanoma Who Were Receiving New Polyvalent Melanoma Vaccine Compared With Those Who Received Prior Tumor Cell Vaccine*

	New Melanoma Cell Vaccine		Old Tumor Cell Vaccine	
	N	%	N	%
Site of first metastases				
Lung	27	36%	28	39%
Skin, nodes	29	39%	19	27%
Brain	5	7%	11	15%
Liver	5	7%	5	7%
Bone	3	4%	6	8%
Gastrointestinal tract	6	8%	2	3%
Other	0	0%	1	1%

Among the 187 patients whose first metastases were regional (AJCC stage IIIA), 61 patients received the new MCV. Among the 1350 patients with distant metastatic sites of recurrence (AJCC stage IV), 75 received the new polyvalent MCV, and 72 were treated with a prior tumor cell vaccine (TCV) as previously described.[7] The remaining patients in the historical control groups with regional and distant metastatic disease received treatment by a variety of methods described above. Table 2 compares the clinical characteristics of those patients receiving the new MCV with those who received other types of treatments for their first site of metastases. Table 3 compares the site of first metastases among AJCC stage IV patients receiving the new MCV with that of those who received the old TCV.

Treatment protocol. Our active specific immunotherapy protocol involved immunization of melanoma patients with a polyvalent, irradiated whole cell MCV. The patients were stratified by stage and disease status and randomized to receive either MCV alone or MCV plus one of the biologic response modifiers, which have been shown to downregulate suppressor cell activity.[30–36] These biologic response modifiers include cimetidine (1200 mg/day) (Smith/Kline, Philadelphia, PA); indomethacin (150 mg/day) (Lederle, Wayne, NJ); and low-dose cyclophosphamide (75, 150, or 300 mg/m^2) (Mead/Johnson, Princeton, NJ).

The new MCV consisted of three human melanoma cell lines (M10, M24, and M101), which were selected from a series of melanoma cell lines after careful examination for the high expression of MAA immunogenic in melanoma patients (Table 1), grown and prepared for administration as previously described.[36] Melanoma cell vaccine was produced in large batches and analyzed for MAA expression to determine variance between lots. An outside laboratory screened the MCV for viral (HIV, hepatitis), bacterial, and fungal infectious organisms. Equal amounts of each line were pooled to a total of 24×10^6 cells in serum-free medium containing 10% dimethyl

sulfoxide and were cryopreserved in liquid nitrogen.[36] Before cyropreservation, the cells were irradiated to 100 Gy.

The MCV was thawed and washed three times in phosphate-buffered saline before administration. Melanoma cell vaccine was injected intradermally in axillary and inguinal regions on a schedule of every 2 weeks × 3, then monthly for 1 year. For the first two treatments, MCV was mixed with BCG (Glaxo, England) (24×10^6 organisms/vial). Since 1989, we have used Tice strain BCG (8×10^6 organisms), due to non-availability of Glaxo BCG. After one year, the immunization interval was increased to every 3 months × 4, then every six months. Follow-up clinical and laboratory evaluations were repeated monthly, with chest x-rays every 3 months.

Laboratory Evaluation

To evaluate the humoral antibody and cell-mediated immune response to MCV therapy, patients were evaluated before and at monthly intervals after immunization. The following assays were performed:

Humoral immune response. The antibody response to melanoma cell surface antigens after MCV immunization was evaluated by the indirect membrane immunofluorescence assay as previously described.[4,22] Sera were tested against the M-14 melanoma after preabsorption with matched lymphoblastoid cells autologous to the test melanoma line to remove antibodies to HLA antigens.[37,38] M-14 expresses all of the six immunogenic MAA at moderate to high levels on its cell surface (Table 1). Similar assays were run against autologous melanoma cells when available. The autologous melanoma was prepared by mincing and enzymatic digestion as previously described[39,40] and placed in short-term culture for 2 to 4 days in RPMI 1640 containing 5% human umbilical cord sera before testing in the indirect membrane immunofluorescence assay.

Delayed cutaneous hypersensitivity. Intradermal skin tests with MCV were performed before and during therapy. One tenth of the pooled MCV (2.4×10^6 cells) was administered at a remote site on the forearm. After 48 hours, the average diameter of the induration was recorded as the delayed cutaneous hypersensitivity (DCH) response. The Student's t test was used to compare the absolute values of DCH from weeks 0 to 4 and to 16.

General immunocompetence was evaluated by sensitization and challenge to dinitrochlorobenzene and response to common skin test antigens such as mumps and candida.[41] The responses to purified protein derivative antigen, to which the patient became sensitized as a result of immunization with BCG in the vaccine, served as additional controls.[41]

Mixed lymphocyte tumor cell reaction. Mixed lymphocyte tumor cell reaction (MLTR) was used to evaluate the *in vitro* response to immunization. Forty patients were selected on the basis of their treatment (MCV alone = 10, plus cimetidine = 11, plus indomethacin = 10, plus cyclophosphamide = 9) and the availability of peripheral blood lymphocyte (PBL) serial bleeds, without knowledge of their clinical condition. Peripheral blood lymphocytes from weeks 0, 4, and 16 were isolated and cryopreserved as previously described.[36] Assays were performed on cryopreserved lymphocytes to ensure reproducibility. Serial bleed PBL were simultaneously thawed, washed, and resuspended in culture medium (RPMI 1640 with 10% human AB serum [heat-inactivated; Irvine Scientific, Santa Ana, CA]).

Each melanoma cell line in the MCV was prepared according to the procedure for vaccine production. Peripheral blood lymphocytes from weeks 0, 4, and 16 were stimulated at a 5:1 ratio to each of the MCV lines (M10, M24, and M101). These co-cultures were performed in triplicate in 96-well microplates with culture medium supplemented with recombinant interleukin-2 20 U/ml (Amgen, Thousand Oaks, CA) to a total volume of 200 μl. They then were incubated for 6 days at 37 C, as previously described.[42] Respective control cultures of PBL were grown in medium alone and with phytohemagglutinin (PHA) (Wellcome, NJ) at a suboptimal concentration of 0.1 μg/mL.[33] During the last 18 hours, the cells were pulsed with [3H]-thymidine (New England Nuclear, Boston, MA) and harvested.[42] Data were analyzed for each patient as counts per minute for each triplicate (standard deviation < 15%) at each time point. The Student's t test was used to compare data from week 0 with that from weeks 4 and 16 for each patient and for the overall study group using mean counts per minute.[43] Each patient served as his or her own control.

Autologous MLTR. Autologous melanoma cells were established from patient biopsy specimens, as described above. These were obtained before therapy and, when available, were assessed with the MCV lines in the MLTR.

Analysis of tumor-infiltrating lymphocytes in melanoma biopsy specimens. Tumor-infiltrating lymphocytes (TIL) have been found in melanoma lesions and are strongly implicated as playing a major role in inducing tumor regression after adoptive immunotherapy. In this study we evaluated TIL in melanoma lesions surgically removed from patients before MCV treatment and compared them by immunohistopathology and flow cytometry with TIL in melanoma lesions removed after MCV treatment from those patients with evaluable lesions at the start of immunotherapy. The immunohistology and histopathologic examinations were carried out by observers blinded to whether the specimens were obtained before or after MCV immunotherapy.

To evaluate lymphocyte subsets, tumor specimens were made into single-cell suspensions by mincing and enzyme

treatment.[40] The single-cell suspensions then were stained with monoclonal antibodies CD3 (T cell), CD4 (T helper), CD8 (T cytotoxic/suppressor), CD19 (Pan B), CD25 (IL-2 receptor, TAC), and CD56 (NK) (Becton Dickinson, Mountain View, CA) specific to individual lymphocyte subsets. The specific binding of CD marker antibodies to cell-surface antigens was then analyzed by flow cytometry using FACscan (Becton Dickinson).

Statistical methods. Estimated survival rates were obtained by the nonparametric Kaplan-Meier method.[44] The log-rank test was used to determine the differences in survival of patients from subgroups defined by different levels of risk factors. This method of univariate analysis is useful when all variables are categorized into subgroups that are maximally separated in terms of survival rates. For example, when the location of the metastatic site is examined, if the survival rate of patients with skin metastases is not statistically different from that of patients with gastrointestinal metastases, but is statistically different from that of patients with lung metastases, the first two groups are combined and compared with the third. This technique was used in part to define categories for the risk factors related to sites of first metastasis. A more general rank test and the log-rank test were used to test for multivariate associations of the risk factors. A discussion of these tests can be found in Kalbfleisch and Prentice.[45] The multivariate results were confirmed by using the Cox proportional hazards regression model.[46] The statistical package of SAS procedures LIFETEST and PHGLM[47] were used in the univariate and multivariate analyses.

Survival time was defined as the time a patient remained alive after the documented date of metastatic disease to either a regional site (AJCC stage IIIA), in regards to skin and soft tissue metastasis, or a distant site (AJCC stage IV). The distant sites were further characterized by the site of the metastasis, as given in Table 2.

Results

Evaluation of the Immune Response to Active Immunotherapy With MCV

A detailed analysis of the humoral antibody and cell-mediated immune response to immunotherapy is beyond the scope of this report. Briefly, most patients demonstrated evidence of a prompt response. A specific anti-melanoma immune response was often induced within 2 weeks, reaching a peak response in 4 to 8 weeks, and gradually declining in most patients to a level significantly above preimmunization levels. As shown below, it is clear that the extent to which the humoral and cell-mediated immune responses are enhanced in these patients correlates with a favorable outcome.

Humoral immune response. We found that IgM antibody to cell surface antigens correlated best with survival, as previously discussed.[22] No significant correlation with IgG antibody to melanoma cell surface antigens was found. As shown in Table 4, the induction of IgM antibodies to membrane-associated melanoma antigens found on the M-14 melanoma was observed in 62% of patients immunized with the new MCV. This was significantly improved over the 35% response to our previous TCV.[7,22] Autologous melanoma cells were available for use as targets in tests of 26 patients receiving the MCV. We found that four of 26 patients had pre-existing antibody to autologous melanoma cells at titers of <1:10 before active immunotherapy with the MCV. After immunotherapy, 17 of 26 patients exhibited antibodies to membrane-associated autologous melanoma antigens to a mean titer of 1:29. This was similar to the observed response to membrane antigens on the allogeneic (M-14) melanoma cells. It should be noted that M-14 melanoma is not a component of the melanoma vaccine, but that parallel rises in antibody to autologous melanoma cells and M-14 were observed in most patients. These data clearly indicate the sharing of common MAA among the MCV and the autologous melanoma cells, thus confirming our previous observations with the humoral response to allogeneic MCV.[22]

TABLE 4. *Comparison of the IgM Antibody Response to Membrane-associated Antigens on Autologous and Allogeneic Melanoma Cells After Active Immunotherapy With an Allogeneic Melanoma Vaccine*

Serum Tested for IgM Antibody*	Target Cell Lines	No. Positive/ No. Tested†	Mean Titer
Preimmunization	M-14 allogeneic	0/26 (0%)	<1:10
Postimmunization	M-14 allogeneic	16/26 (62%)	1:38
Preimmunization	Autologous	4/26 (15%)	<1:10
Postimmunization	Autologous	17/26 (65%)	1:29

* Sera were preabsorbed with L-14 lymphoblastoid cells, which are autologous to the M-14 melanoma, to remove antibodies to HLA antigens before immunofluorescence assays.[4,37,38]

† Positive defined as those sera exhibiting an MIF index of >0.20.

MIF, membrane immunofluorescence.

High levels of anti-melanoma antibodies with membrane immunofluorescence indices of >50% were associated with significant ($p < 0.01$) improvement in survival, as illustrated in Figure 1 and previously reported with our prior TCV.[22] We observed almost a threefold increase in 5-year survival (9.6% to 26.8%) and a twofold increase in median survival from 16 to 30 months among the high responders.

Delayed cutaneous hypersensitivity. Most patients were judged generally immunocompetent by their response to purified protein derivative, dinitrochlorobenzene, or common skin test antigens.

When all patients were stratified by their maximum skin test reactions to MCV, there was a highly significant ($p = 0.0066$) correlation between survival after treatment

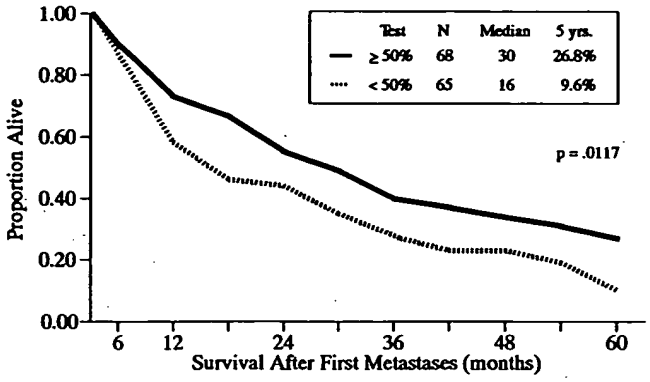

FIG. 1. Correlation between IgM antibody response and survival in patients with advanced-stage melanoma who were receiving active immunotherapy. Five-year survival rate 26.8% *versus* 9.6%.

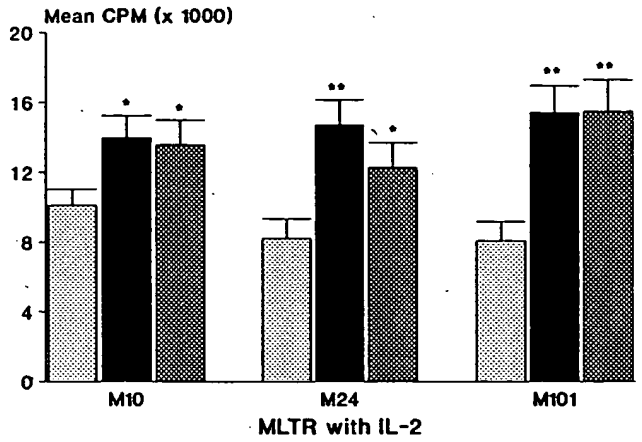

FIG. 3. Stimulation of MCV patients' PBL to MCV lines M10, M24, and M101 in MLTR plus IL-2 at week 0 (▨), week 4 (■), and week 16 (▩). Data expressed as mean CPM (±SEM) from [^{3}H]-thymidine incorporation assay. Significance determined by Student's t test of mean CPM for each patient from week 0 to week 4 (n = 40) and week 0 to week 16 (n = 37). *p < 0.05; **p < 0.001.

and a DCH reaction > 10 mm during the first 12 weeks after initial MCV therapy. The median survival was 30 months for those >10.0 mm and only 17 months for those <10.0 mm (Fig. 2). Five-year survival increased from 10% to 27.7%.

Mixed lymphocyte tumor reactions. To evaluate the cellular immune response of *in vivo* stimulation with MCV, MLTRs were performed with the individual MCV lines in 40 patients. Overall, MLTR with M10, M24, and M101 were significantly increased at week 4 compared with week 0, and the level of response to each MCV line remained significant at week 16 (Fig. 3). The patterns and magnitude of the patients' responses were similar in MLTR performed without the presence of IL-2 (data not shown). A comparison of the *in vivo* DCH response to the MCV and the *in vitro* MLTR in the 40 patients for whom there was data on both cell-mediated assays shows similar patterns and magnitudes of responses (data not shown).

Of the 40 patients, 82% showed significantly ($p < 0.05$) enhanced stimulation to one or more of the MCV lines at either week 4 or 16 compared with week 0. Of these, 91% had evidence of sensitization to at least two MCV lines. More patients showed sensitization at week 4 to M24 and M101 (73% and 75%, respectively) than to M10 (38%), and more maintained sensitization at week 16 to M24 and M101 (51% and 62%, respectively) than to M10 (35%).

The proliferation of PBL in medium alone, and with PHA, was assessed at weeks 0, 4, and 16. Overall, at each time point, PBL response was significantly ($p < 0.05$) greater with PHA than in medium alone. More importantly, comparisons from weeks 0 to 4 and 16 showed no significant differences, either with medium alone or with PHA. These and additional controls (data not shown), along with the variable responses to the MCV lines, indicated that the MLTR responses were not the result of nonspecific responses to cryopreservation, culture medium, serum antigens in the AB serum, or preparation procedures.

Autologous MLTR. To determine whether patients become sensitized to their own melanomas as well as to the allogeneic MCV cells during MCV immunotherapy, autologous melanoma MLTR were performed in parallel with MLTR against MCV vaccine lines. Figure 4A and 4B shows two representative patients who demonstrated significant sensitization toward their own melanoma at weeks 4 and 16 compared with week 0. Patient 21 (Fig. 4B) had evidence of pretreatment sensitization to his own melanoma, which was significantly augmented during MCV therapy. Thus, immunization with this allogeneic melanoma vaccine clearly enhanced response to the autologous melanoma, confirming the observations of cross-reacting antigens, seen by humoral antibody to membrane-associated antigens (Table 4).

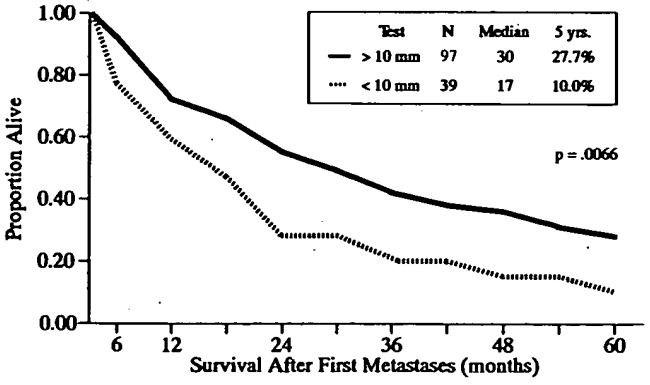

FIG. 2. Comparison of the response to MCV skin tests and survival in advanced-stage melanoma. Five-year survival rate 27.7% *versus* 10%.

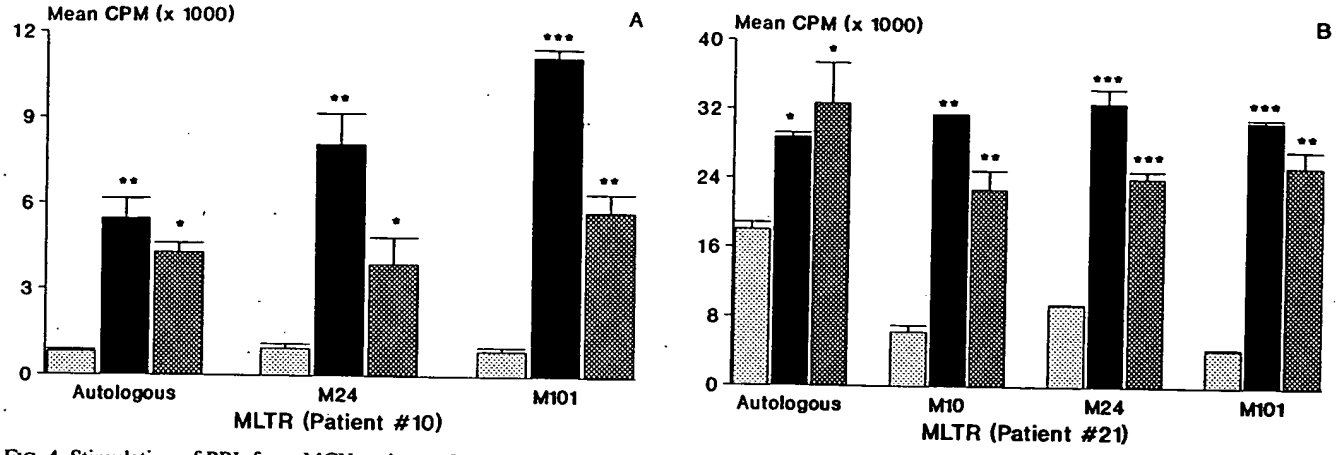

FIG. 4. Stimulation of PBL from MCV patients 10 (A) and 21 (B) to autologous melanoma and MCV lines at week 0 (▨), week 4 (■), and week 16 (▩) in parallel MLTR plus IL-2. Data expressed as mean CPM (±SEM) from [^{3}H]-thymidine incorporation assay. Comparisons from week 0 to week 4 and week 0 to week 16 by Student's t test of CPM. No significant differences were noted to M10 (data not shown) in patient 10. Patient 21 had evidence of pretreatment (week 0) sensitization to his own tumor, but not to the MCV lines. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The nature of the antigens to which the lymphocytes in the MLTR react is complex and probably involves MHC (major histocompatibility complex) class I or II antigens, as well as specific MAA. There was a clear-cut correlation between response in MLTR and survival, as discussed below. Furthermore, the parallel responses of autologous melanoma cells (Fig. 4A and B) indicate that antigens other than MHC are involved in the MLTR assays.

MLTR correlation with clinical data. Of the 40 patients, 37 were clinically NED at the start of treatment; 28 were stage IIIA; and nine were stage IV. At the time of analysis, 25 of 37 (68%) were alive, with a mean follow-up of 26 months (range, 14 to 50 months). Of these, 16 remained NED, five had had surgical resection of a recurrence and were REC-NED, and four had recurrent disease with progression and were AWD. Twelve patients, 10 stage IIIA and two stage IV, died (mean, 19 months; range, 8 to 32 months from time of treatment).

Disease-free and overall survival for the 37 patients who began treatment NED were correlated with the individual patient's response to MCV by MLTR. Week 16 was selected as the evaluation point based on the number of vaccinations (n = 5).

The disease-free survival at 2 years was 53% ± 10% standard error of the mean (SEM) for patients responding to one or more MCV lines in the MLTR, compared with 20% ± 13% SEM for patients who showed no response; the difference between the two groups approached significance ($p = 0.055$). In the responding patients, the median time to recurrence was > 29 months, compared with 12 months in the nonresponders. The overall survival rate at 2 years was 78% ± 9% SEM for the responding patients, compared with 50% ± 16% SEM for the nonresponders. Again, the difference between the two groups approached significance ($p = 0.065$). The median survival was >36 months in the responders, compared with 20 months in the nonresponders.

Analysis of TIL in patients receiving active immunotherapy. A detailed analysis of the histopathology of melanoma biopsy specimens of patients receiving active immunotherapy showed an increase in intratumoral lymphocytes infiltrating the melanoma cells. This phenomenon is illustrated in Figure 5A and B by hematoxylin and eosin staining of a biopsy of a post-MCV pulmonary metastasis. Figure 5C indicates that the lymphocytes in this specimen are primarily T cells (pan-T staining). Little staining was observed with a Pan B stain (not shown). In addition to the findings regarding TILs, there was an increase observed in the postimmunization biopsies of both single-cell necrosis (defined as isolated necrotic cells surrounded by lymphocytes) and confluent necrosis, indicated by sheets of necrotic cells.

An interesting observation in the patients receiving MCV was the appearance of peripheral lymphoid aggregates surrounding melanoma metastases in the subcutaneous tissues (Fig. 6A). These aggregates contained both T and B cells (Fig. 6B and C), which appeared to be organized into lymphoid follicles.

To confirm further our findings regarding changes in postimmunization specimen histopathology, we undertook a study of preimmunization and postimmunization biopsies to evaluate changes in lymphocyte subsets of the TILs by flow cytometry using FACscan. The results are summarized in Table 5, where we show the analysis of five pre-MCV biopsies and nine post-MCV melanoma biopsies for specific TIL subsets. A comparison of pre-MCV *versus* post-MCV biopsies showed an increase in the CD4+/CD8+ ratio of the postimmunization biopsies, from a mean of 0.93 to 2.13. Although they were not

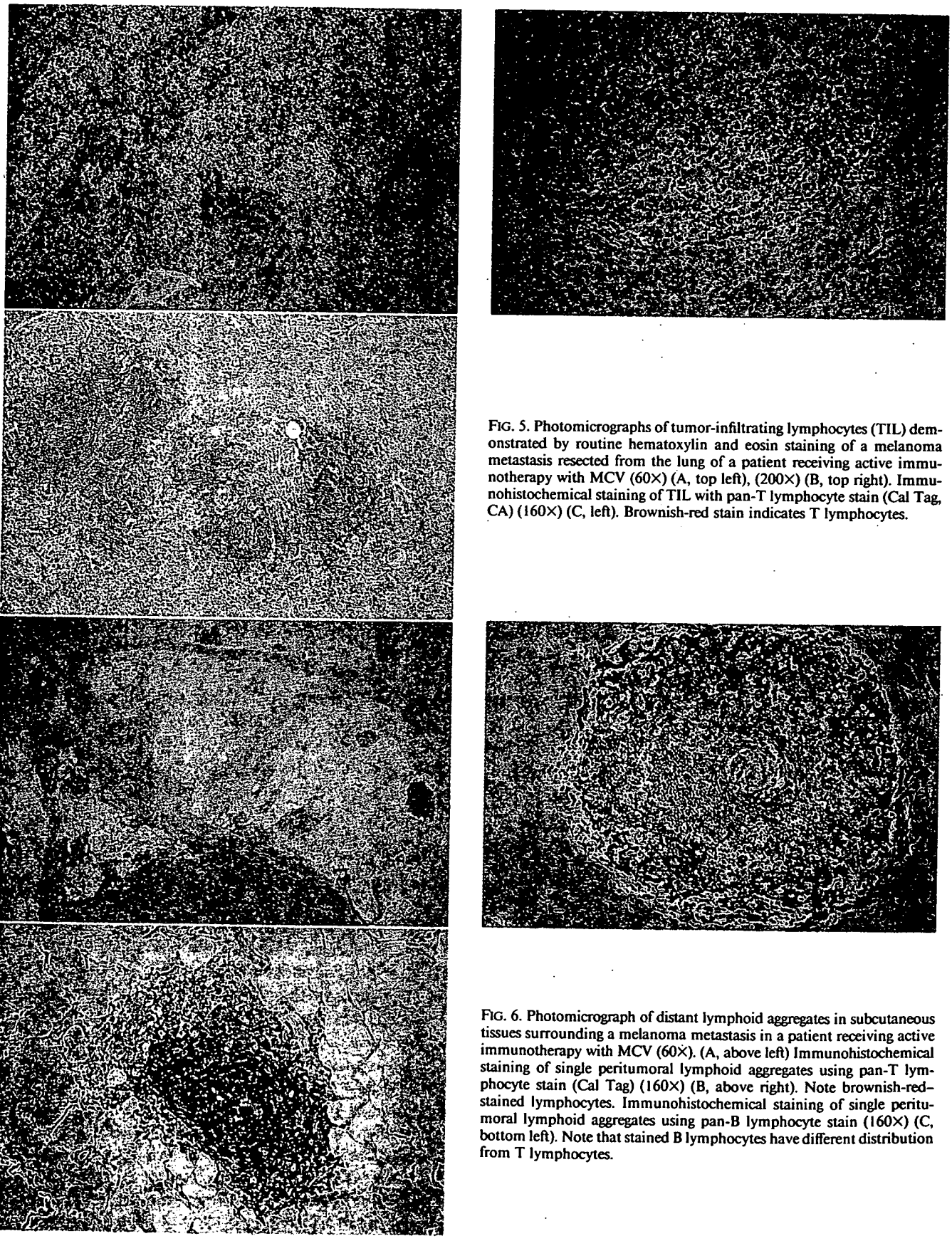

FIG. 5. Photomicrographs of tumor-infiltrating lymphocytes (TIL) demonstrated by routine hematoxylin and eosin staining of a melanoma metastasis resected from the lung of a patient receiving active immunotherapy with MCV (60×) (A, top left), (200×) (B, top right). Immunohistochemical staining of TIL with pan-T lymphocyte stain (Cal Tag, CA) (160×) (C, left). Brownish-red stain indicates T lymphocytes.

FIG. 6. Photomicrograph of distant lymphoid aggregates in subcutaneous tissues surrounding a melanoma metastasis in a patient receiving active immunotherapy with MCV (60×). (A, above left) Immunohistochemical staining of single peritumoral lymphoid aggregates using pan-T lymphocyte stain (Cal Tag) (160×) (B, above right). Note brownish-red–stained lymphocytes. Immunohistochemical staining of single peritumoral lymphoid aggregates using pan-B lymphocyte stain (160×) (C, bottom left). Note that stained B lymphocytes have different distribution from T lymphocytes.

TABLE 5. *Tumor-infiltrating Lymphocyte Phenotype Analysis of Melanoma Biopsy Specimens Before and After Melanoma Cell Vaccine Immunotherapy*

Patient	% Staining						
	CD19	CD3	CD4	CD8	CD4/CD8	CD25	CD56
Lymphocyte phenotype of TIL before MCV							
A	5	89	12	46	0.26	0	0
B	58	34	23	34	0.79	1	3
C	19	72	37	17	2.17	2	1
D	43	50	36	47	0.76	1	2
E	21	63	28	41	0.68	2	3
Mean	29	62	27	37	0.93	1	2
Lymphocyte phenotype of TIL after MCV							
C	42	46	30	9	3.33	12	7
E	40	56	36	31	1.16	1	1
F	36	56	39	19	2.05	6	5
G	33	48	26	16	1.62	1	8
H	3	84	26	49	0.53	8	9
I	62	38	23	6	3.83	4	0
J	54	37	26	13	2.00	4	8
K	18	73	26	57	0.46	3	7
L	35	43	38	9	4.22	4	15
Mean	36	53	30	23	2.13	5	7
P*	<0.6	<0.4	<0.6	<0.2	0.10	<0.05	<0.04

* Comparison of pre- *versus* post-MCV treatment.
TIL, tumor-infiltrating lymphocyte; MCV, melanoma cell vaccine.

statistically significant ($p = 0.10$), the results show a strong trend in the reduction of CD8+ T cells (most likely suppressor T cells) in post-MCV biopsies. In one of the pre-MCV treated specimens (patient C), the CD4+/CD8+ ratio was unusually high. In the same patient's post-MCV–treated specimen, this ratio was further enhanced. Similar changes were noted in the pre-MCV and post-MCV specimens of patient E. Patient C's post-MCV specimen also had a high level of activated TILs with IL-2 receptors (CD25+). In the other post-MCV specimens there was an overall statistically significant enhancement of CD25+ ($p < 0.05$) and CD56+ ($p < 0.04$) cells. The presence of a higher level of CD25+ cells in the post-MCV specimens indicates an increased level of lymphocyte activation, which is consistent with the findings of immunohistopathology. Although it would have been preferable to have matched specimens for comparison of pre-MCV and post-MCV in the same patients, this was not always possible. However, both specimens were available for flow cytometric analysis for patients C and E. Data from these two patients and the paired specimens from many more patients that were available for histopathology study confirmed the changes noted in random flow cytometry studies of preimmunization and postimmunization biopsies.

The overall findings indicate that active specific immunotherapy with our new polyvalent allogeneic melanoma vaccine appears to be followed by enhanced activation of specific lymphocyte subsets within the melanoma specimen. These lymphocytes appear to migrate to the site of melanoma metastases and may be responsible for the complete and partial melanoma regressions, as well as the delayed progression observed after active immunotherapy.

Univariate and Multivariate Analysis of Prognostic Factors

As shown in Figure 7, patients with AJCC stage IIIA regional soft tissue metastases survived significantly ($p = 0.0001$) longer than those with distant metastases (AJCC stage IV). Therefore, it was necessary to analyze those two stages separately when comparing survival in patients being immunized with the new MCV. Table 6 lists the factors analyzed by univariate and multivariate analysis. Only two factors were significant for prognosis in stage IV melanoma. One was the first site of distant metastasis, with the skin, subcutaneous, gastrointestinal, and nodal sites being the most favorable; lung and bone being intermediate; and the liver and brain being the least favorable. The other major prognostic factor that was highly significant was whether or not the patients received immunotherapy with our new MCV. Those patients who received the new MCV survived significantly longer than those who were treated by other means. Unlike Balch et al.,[1] we did not find the remission duration or number of metastatic sites to be of prognostic significance by multivariate analysis. As shown in Table 2, however, the number of metastatic sites was closely matched in the MCV and historical control groups.

Analysis of the Influence of the Chronologic Time Interval of Treatment on the Survival of Patients With Metastatic Melanoma

Because the chronologic time interval of treatment could have been an important factor in the survival of the historical control patients, we divided the patients re-

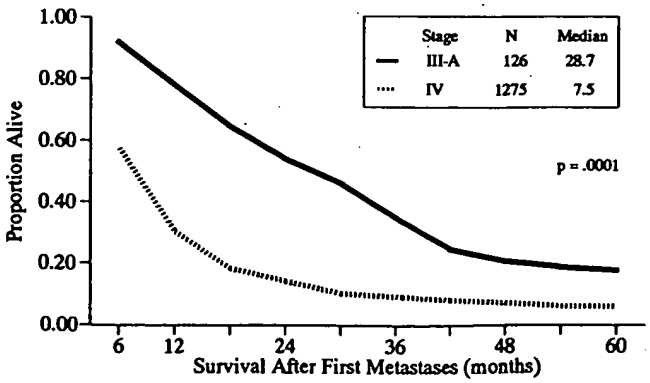

FIG. 7. Comparison of the survival of 126 melanoma patients with regional soft tissue metastases (AJCC stage IIIA) with 1275 patients with distant metastases (AJCC stage IV) who were treated during the period from 1971 to 1991.

TABLE 6. *Analysis of Prognostic Factors by Univariate and Multivariate Methods for Stage IV Melanoma*

Factor	p	
	Univariate	Multivariate
Sex	0.0824	0.2450
Age	0.9224	—
Extremity *vs.* nonextremity	0.5290	—
Breslow thickness <1.5 *vs.* >1.5	0.1172	—
Clark depth	0.1240	—
Remission time before metastases	0.2948	0.3532
No. of metastases	0.0790	0.7775
Site of metastases	0.0001	0.0001
Immunotherapy with melanoma vaccine	0.0001	0.0001

ceiving other treatments into three groups of 6- or 7-year periods to determine whether there had been an improvement in survival of patients in the other treatment groups that might explain the improved survival observed in our most recent patients receiving the new MCV. Figures 8 and 9 give the survival rates during various time intervals for patients with AJCC stage IIIA and AJCC stage IV disease who received other treatments. As can be seen, there has been no improvement in the survival of patients with metastatic melanoma who received non-MCV therapy and were seen by the staff of the JWCI during the past 20 years. This is not surprising because the standards of care for patients with metastatic melanoma have remained very much the same during the past 20 years, and there has been little improvement in survival observed with different chemotherapy regimens. Thus, the improved survival of patients receiving the new MCV cannot be related to chronologic differences in the time frame in which these patients were treated.

Comparison of Overall Survival Between Active Immunotherapy and Historical Control Groups

As shown in Figures 10 and 11, there was a highly significant improvement in the survival of patients with both

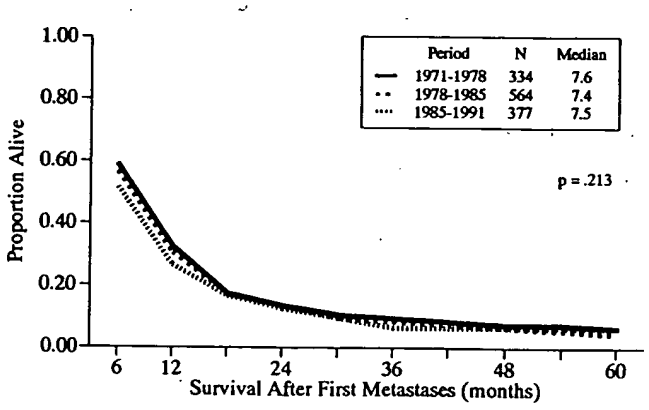

FIG. 8. Comparison of the survival of 1275 patients with AJCC stage IV melanoma by time interval of treatment over the period 1971 to 1991. Five-year survival rate, 7.0% *versus* 5.0% *versus* 7.0%.

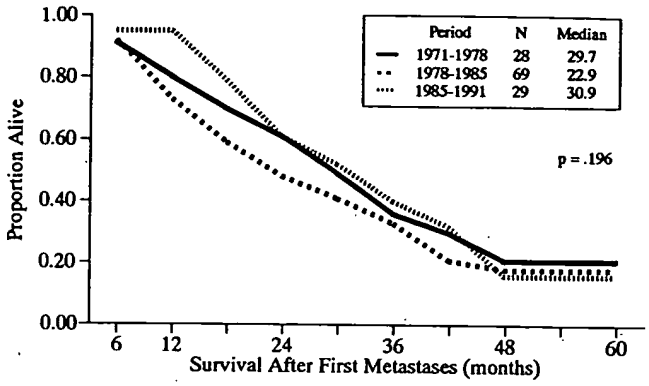

FIG. 9. Comparison of the survival of 126 patients with AJCC stage IIIA melanoma by time interval of treatment over the period 1971 to 1991. Five-year survival rate, 21% *versus* 18% *versus* 16%.

AJCC stage IIIA and stage IV disease who received active immunotherapy with the new polyvalent MCV. The median survival of stage IV was increased threefold from 7.5 to 23.1 months, and 5-year survival was increased fourfold, from 6% to 26%.

The distribution in Table 2 of patients receiving other therapy, however, differs from that of those receiving immunotherapy: the "other" patients were slightly more likely than patients receiving the new MCV to have brain (9%) and liver (7%) metastases and less likely to have skin or subcutaneous metastases (18%), whereas patients receiving the old TCV showed a distribution of metastatic sites that was quite similar (within 8% to 12%) to that of patients receiving the new vaccine (Table 3). It is unlikely that such small differences in distribution of metastases could be responsible for such large differences in survival between the two groups of patients with stage IV disease.

Multivariate analysis took into account the differences in risk factors in comparing the two survival curves; however, we thought it important to exclude the possibility

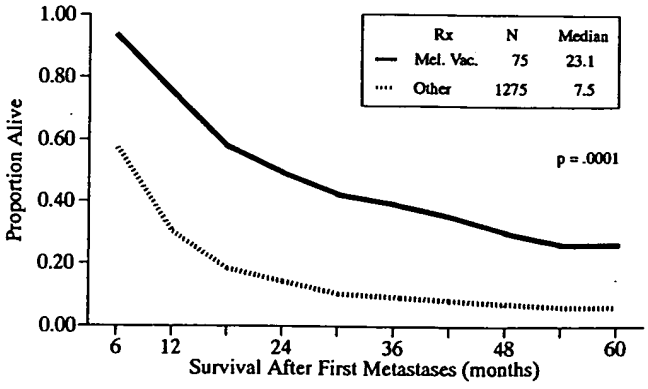

FIG. 10. Comparison of the survival of 75 patients with AJCC stage IV disease who received active immunotherapy with the new MCV *versus* 1275 historical controls who received other types of therapy. Five-year survival rate, 26% *versus* 6%.

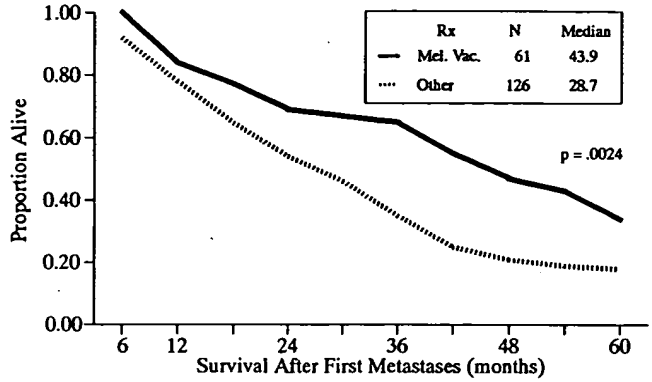

FIG. 11. Comparison of the survival of 61 patients with AJCC stage IIIA disease who received immunotherapy with the new MCV *versus* 126 historical controls who received other types of therapy. Five-year survival rate, 34% *versus* 18%.

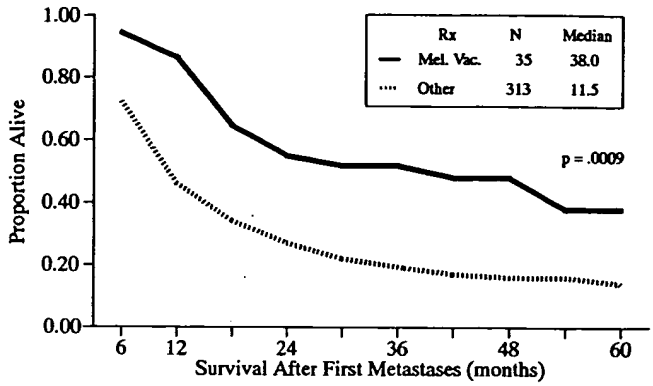

FIG. 13. Comparison of the survival of 35 patients with AJCC stage IV melanoma and soft tissue metastases who received active immunotherapy with the new MCV *versus* 313 historical controls who received other types of therapy. Five-year survival rate, 38% *versus* 14%.

of bias due to a more favorable pattern of metastatic sites being present in the patients receiving immunotherapy with the new vaccine. Therefore, we directly compared the two groups of patients who were treated either with the new MCV or with other treatments for three metastatic sites: lung (Fig. 12); soft tissue (skin, subcutaneous, and nodal sites) (Fig. 13); and liver and brain (Fig. 14). Again, we found that patients receiving the new vaccine survived significantly longer than those patients treated by other methods. Furthermore, as is shown in Figure 15, the patients receiving the new MCV demonstrated a highly significant improved survival compared with those receiving the old TCV.

Finally, we investigated a possible bias due to the fact that the patients who had received the new melanoma vaccine had been more recently entered into the trial. Because many of them were still alive, their data was censored. This may have created a bias when compared with the other data sets in which more of the patients had died. To investigate this possible bias, we compared the survival rates of the two groups of patients by comparing only those patients who had died of melanoma in the subsets of patients with lung metastases or skin and subcutaneous metastases (Fig. 16A and B). Again, there was a highly significant improvement in survival for both sites in those patients who had received immunotherapy with the new vaccine as compared with the patients treated by other therapies. Thus, it is clear that censoring cannot explain the apparent increased survival rate in those patients receiving the polyvalent MCV.

Clinical Results in Patients With Evaluable Disease at the Time of Initiation of Immunotherapy

A total of 40 stage IV patients entered the study with evaluable metastatic disease and were observed for 12 weeks after the initiation of immunotherapy. The incidence of complete and partial regression of disease is given in Table 7. Regressions were observed in nine of the 40 patients, an objective regression rate of 23%. Three com-

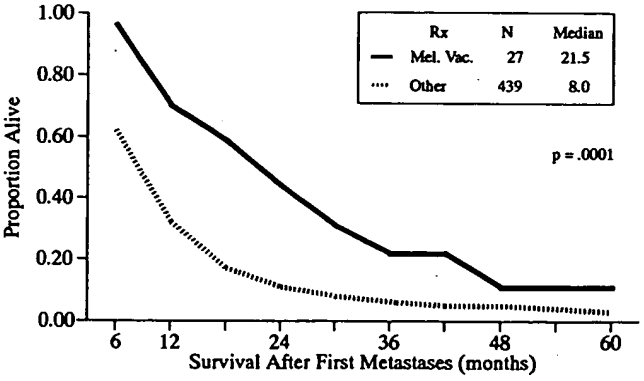

FIG. 12. Comparison of the survival of 27 patients with AJCC stage IV melanoma and lung metastases who received active immunotherapy with the new MCV *versus* 439 historical controls who received other types of therapy. Five-year survival rate, 11% *versus* 3%.

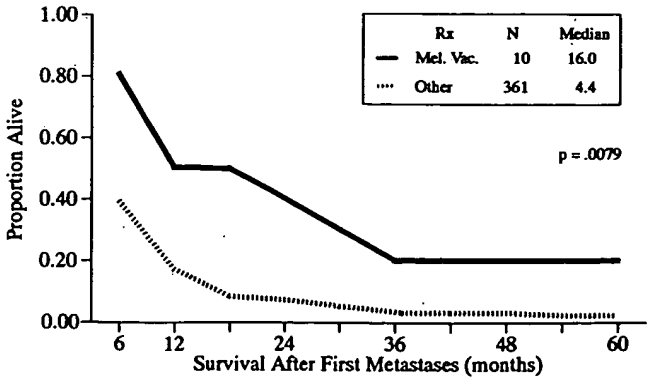

FIG. 14. Comparison of the survival of 10 patients with AJCC stage IV melanoma and liver/brain metastases who received active immunotherapy with the new MCV *versus* 361 historical controls who received other types of therapy. Five-year survival rate, 20% *versus* 2%.

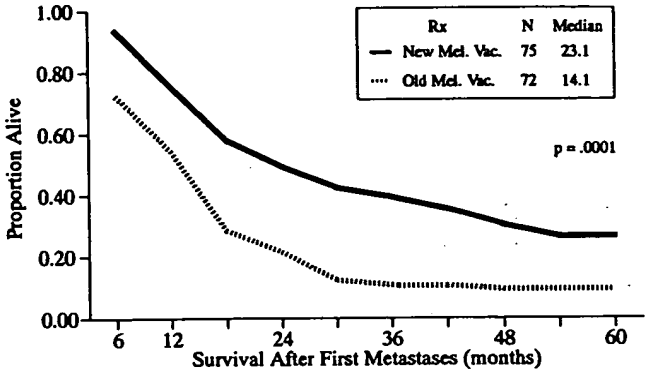

FIG. 15. Comparison of the survival of 75 patients with AJCC stage IV melanoma who received active immunotherapy with the new MCV *versus* the 72 patients immunized with a prior TCV. Five-year survival rate, 26% *versus* 10%.

plete responses and six partial responses were observed. Most patients exhibited progressive disease that required the institution of other therapies, primarily chemotherapy. We sometimes observed stabilization in growth of metastasis, however, and in some patients, such as those with pulmonary metastasis, we could objectively measure the growth rates before and after the beginning of immunotherapy based on the tumor doubling time.[48] In some cases, as shown in Figure 17, there was a clear reduction in the growth rate of the metastatic disease. It was not unusual for patients to observe tenderness and swelling, sometimes accompanied by erythema, pain, or itching, at the sites of melanoma metastases, beginning 2 to 4 days after repeated booster immunizations. One patient exhibited bruising at sites of subcutaneous metastases, followed by complete regression of that particular subcutaneous metastasis. Photographic documentation of two of the three complete responses is given in Figures 18 and 19.

Case 1 (Fig. 18A–D) is a 53-year-old man whose primary melanoma was behind the ear. The primary was treated by wide excision and a radical neck dissection. No lymph nodes were involved, and the patient remained well for 4 years. After 4 years, recurrent in-transit disease developed surrounding the primary despite adjuvant DTIC and BCG. The disease on the head and neck was treated with electron beam radiation with some response, followed by further progression. At the time the immunotherapy was initiated (January 6, 1986), extensive multiple metastases were present over the right cheek, ear, and scalp, posterior to the ear as well as extending to both sides of the neck, as shown in Figure 18A and 18B. Approximately 12 weeks after the initiation of immunotherapy with the polyvalent MCV, using low-dose cyclophosphamide 300 mg/M^2 as an immunomodulator, certain of the patient's metastatic lesions showed some flattening and a reduction in size. During the following 19 months, a complete regression of all metastases occurred (Fig. 18C) concomitantly with continuation of MCV immunotherapy. The anti-melanoma antibody titers to membrane-associated MAA began to increase after 4 weeks, as shown in Figure 18D.

Brain metastases were suspected after a seizure, and the magnetic resonance imaging scan showed a mass lesion, but no melanoma cells were found in specimens after craniotomy and resection, suggesting that immunotherapy had caused regression of the brain metastases. The patient remained in complete remission for an additional year, at the end of which a small recurrent nodule was noted on the ear. Treatment with intralesional injections of human monoclonal antibody[23] produced complete regression of the nodule. The patient was continued on melanoma vaccine and remained well until 4½ years after the onset of the immunotherapy, when he again experienced seizures. Workup then disclosed meningeal spread of his melanoma. After shunt placement for increased intracra-

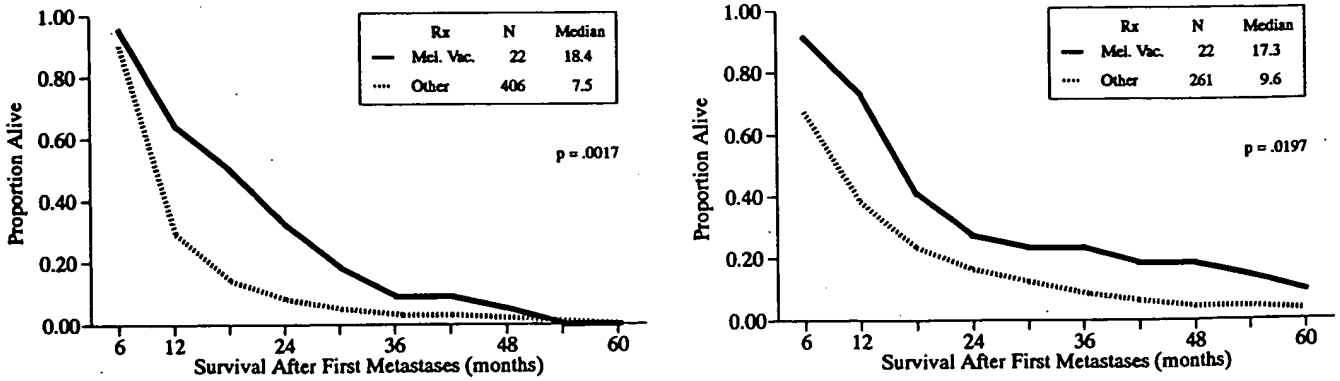

FIG. 16. (A, left) Comparison of the survival of 22 patients with AJCC stage IV melanoma who had fatal lung metastases and received active immunotherapy with the new MCV *versus* 406 historical controls who received other types of therapy. Five-year survival rate 0% *versus* 0%. (B, right) Comparison of the survival of 22 patients with AJCC stage IV melanoma who had fatal soft tissue metastases and received active immunotherapy with the new MCV *versus* 261 historical controls who received other types of therapy. Five-year survival rate, 9% *versus* 2%.

TABLE 7. *Objective Responses in Patients With Evaluable Disease**

Disease	N	%
Complete	3	8
Partial	6	15
Stable	4	10
Progression	27	67
Total	40	100.0

* Measurable disease observed for at least 12 weeks after onset of immunotherapy.

nial pressure, he was treated with chemotherapy using a cisplatin-based regimen, but the patient died 4 months later.

Case 2 (Fig. 19A and B) is a 69-year-old woman with a Clark's level IV melanoma of the thigh, who was treated by a wide excision and a radical inguinal lymphadenectomy. Eighteen months later, she developed multiple satellite metastases around the site of the primary. These were treated with radiation and local hyperthermia, which resulted in a burn at the site of the hyperthermia. Her melanoma continued to progress, with multiple metastatic lesions involving the entire thigh and extending above the inguinal ligament (Fig. 19A). The patient was seen in the John Wayne Cancer Clinic in April 1988, at which time a biopsy of cutaneous metastases showed active melanoma (Fig. 19B). Immunotherapy with the polyvalent MCV was initiated. The disease continued to progress until 8 weeks after the initiation of the new vaccine, when it appeared to stabilize. From 12 to 16 weeks, there was clear evidence of regression in the cutaneous in-transit metastases, and over the next 3 months her disease underwent a complete regression. The actively growing melanoma nodules were replaced by flattened pigmented areas, which have gradually faded (Fig. 19C). Biopsy of these pigmented lesions disclosed no visible melanoma cells, only pigment in macrophages (Fig. 19D). The patient has been maintained on the new MCV every 2 months. She is now 4 years 5 months since the initial recurrence and 4 years 4 months since the initiation of immunotherapy. A recent workup, including full-body computed tomography (CT) scans, showed no evidence of recurrence at any site.

Case 3 is a 40-year-old woman with ocular melanoma who presented with multiple liver metastases visible on a CT scan. Because we did not know whether her ocular melanoma shared cross-reacting antigens with cutaneous melanoma, we carried out an exploratory laparotomy and resected one of the metastases in the left lobe of the liver to obtain tissue for antigenic typing. We found that her ocular melanoma shared most of the antigens with cutaneous melanoma. Cytotoxic T lymphocytes (CTL) generated by stimulation with the patient's melanoma killed HLA-A-matched allogeneic cutaneous melanoma.[49] We therefore proceeded with active specific immunotherapy

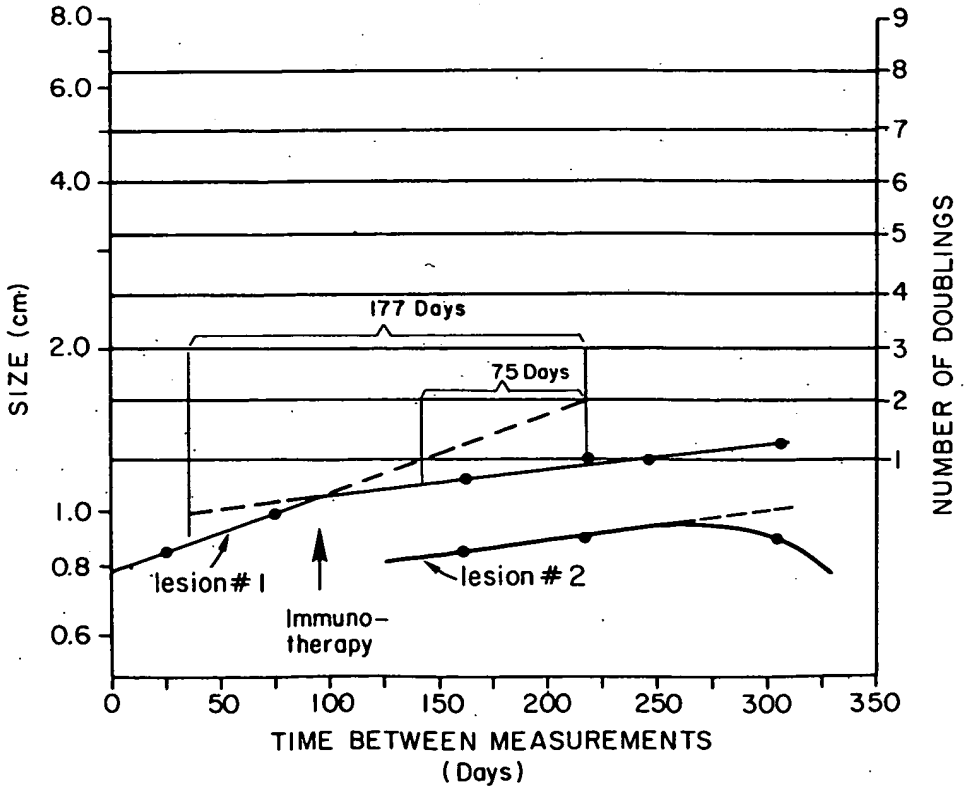

FIG. 17. The slowing of rate of growth, as judged by a change in tumor doubling time (TDT) in a female patient receiving active immunotherapy with the polyvalent MCV. Lesion 1 TDT increased from 75 to 177 days, whereas lesion 2 remained unchanged in size.

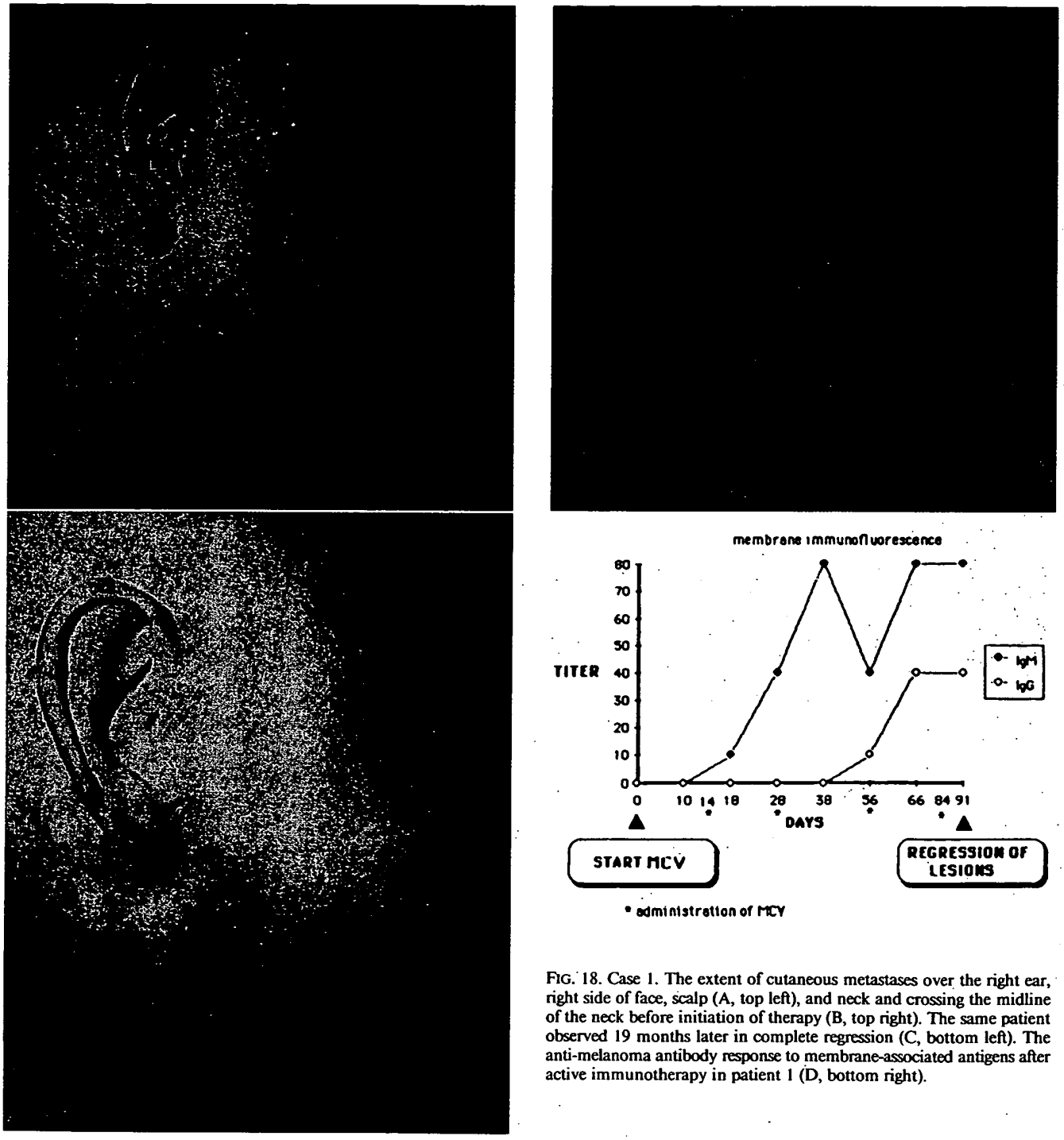

FIG. 18. Case 1. The extent of cutaneous metastases over the right ear, right side of face, scalp (A, top left), and neck and crossing the midline of the neck before initiation of therapy (B, top right). The same patient observed 19 months later in complete regression (C, bottom left). The anti-melanoma antibody response to membrane-associated antigens after active immunotherapy in patient 1 (D, bottom right).

using the polyvalent MCV. A repeat CT scan 3 months after the initiation of immunotherapy showed a 75% regression of the metastatic disease in the right lobe of the liver. The response was maintained for another 3 months, but then the disease began to progress, and chemotherapy with a cisplatin-based regimen was instituted. The patient died 15 months after the initiation of immunotherapy.

Toxicity

Galaxo and Tice Strain BCG at dosages of 8 million and 4 million organisms admixed with the MCV on the first and second treatment cycles cause local erythema, induration, and ulceration at the sites of intradermal administration. This is most notable in tuberculin-positive

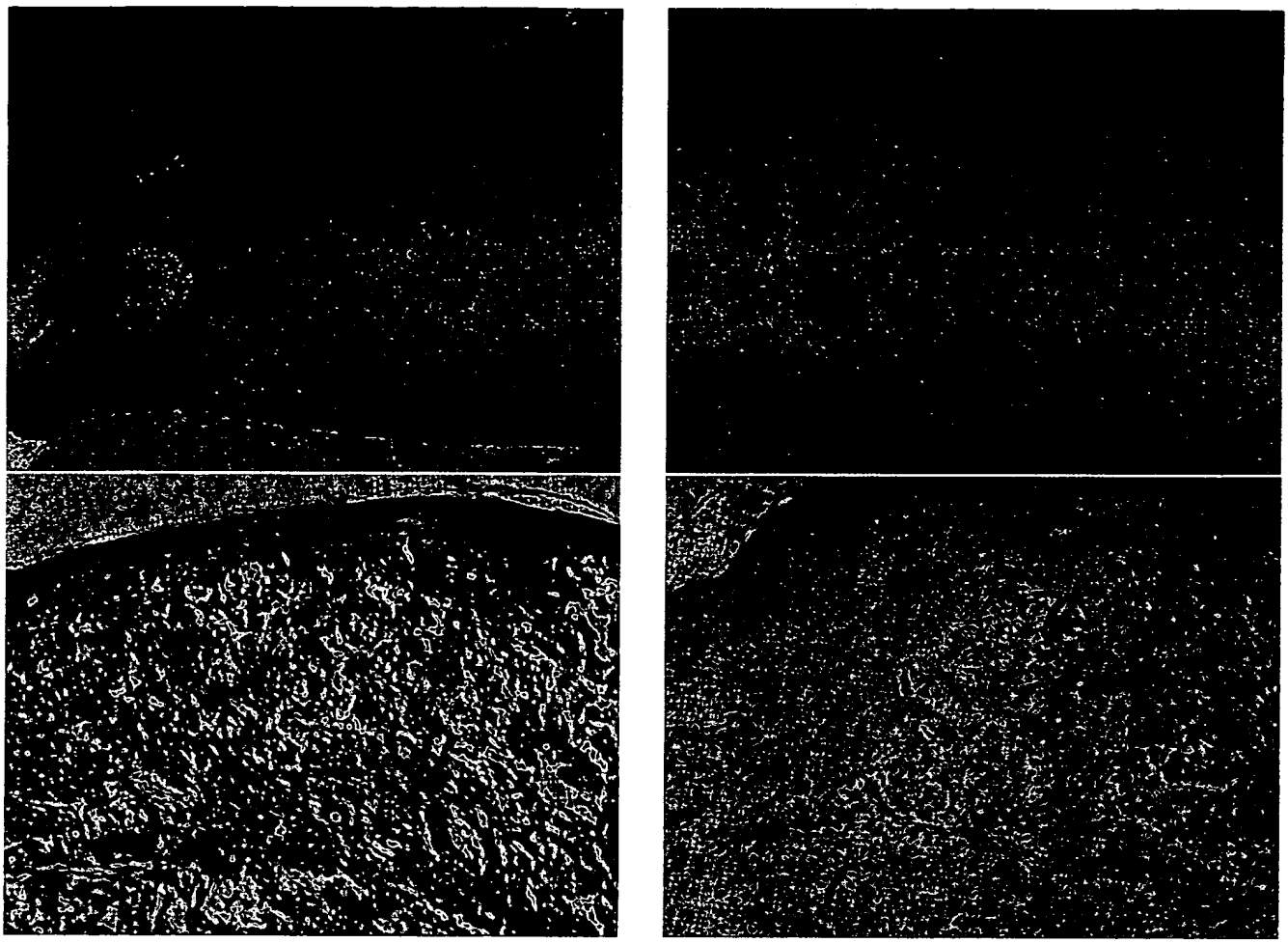

FIG. 19. Case 2 The extent of cutaneous metastases at the time of initiation of immunotherapy. The granulation wound is secondary to a hyperthermia burn (A, top left). Photomicrograph showing actively growing viable melanoma cells (B, bottom left). Flattening and fading of pigmented lesions with complete regression 4 months later (C, top right). Photomicrograph showing pigment in microphages but no viable melanoma cells (D, bottom right).

patients for whom the above dosages are diminished by half. Low-grade fever is noted by about 35% of patients, usually within the first 72 hours after treatment with BCG, sometimes accompanied by myalgia and arthralgia. Fewer than 10% of patients report myalgia, arthralgia, chills, or rigors. These symptoms, when they occur, generally last less than 48 hours and respond to aspirin.

Local ulcerations generally peak by about week 4 of treatment and heal progressively over approximately 8 weeks. The remaining scars are generally modest in degree and tend to fade slowly over 8 to 10 months. In general, both local and systemic toxicity is less than we previously observed with intralesional BCG.[50]

Melanoma cell vaccine, when administered alone, is very well tolerated, with virtually no significant toxicity when administered up to 5 years at 3-month intervals. Mild erythema and itching in the treatment sites are noted by most patients, but these are transient, lasting only 2 to 3 days. About 15% of patients report low-grade fever of <99 F for 12 to 24 hours. A similar proportion of patients report mild fatigue on the day or two after treatment with MCV alone. Myalgia and arthralgia are rarely reported.

Discussion

The concept of using vaccines to induce specific immunity against cancer has existed since the turn of the century, when cancer therapists were first attracted by the success of vaccines in inducing active immunity against infectious diseases. Cancer vaccines differ from vaccines against infectious diseases in that they are administered as therapy after the advent of disease, rather than prophylactically before the disease develops. The theory behind vaccines for cancer and infectious diseases is, however, similar. Both seek to stimulate the patient's own

immune system to fight the disease through the introduction of killed whole organisms or cells, specific subcellular antigens, and nonpathologic strains of living organisms or tumor cells.[51]

Early attempts to induce tumor regression in cancer patients by immunizing them with their own tumors or with those from other patients were not properly evaluated; they lacked suitable controls and immunologic studies to determine whether the patients had actually been successfully immunized. There are well-documented instances, however, of vaccine-induced immunity against cancer in animal models and evidence for serologic and clinical responses in humans to suggest that active specific immunotherapy can be developed as a modality of treatment for cancer. Active immunotherapy became a realistic strategy after it was demonstrated that the induction of DCH reactions in certain malignant neoplasms, such as those induced by the intralesional injection of BCG (an attenuated strain of Mycobacterium),[25] resulted in the regression and eradication of the directly injected cutaneous melanoma metastases and occasionally also in the regression of uninjected metastases. These reports rekindled interest in the concept of a vaccine for cancer and revived efforts to find the crucial formulas for effective vaccine therapy.

Our results demonstrate the ability of the new polyvalent MCV to sensitize patients during the course of immunization. Overall, the humoral antibody response to melanoma-associated membrane antigens and delayed cutaneous hypersensitivity to the MCV and the MLTR show that melanoma patients develop both humoral and cell-mediated immunity to MCV after two MCV treatments and maintain sensitization after five MCV treatments. Parallel *in vivo* assessments of DCH also show significant sensitization in the MCV-treated patients. The correlation of the DCH with the MLTR and the type of responses to the individual MCV lines indicates that the cellular immune responses were amnestic, not inflammatory, or nonspecific.

One of the crucial questions regarding the use of an allogeneic vaccine relates to the question of whether cross-reactive antigens are present on the autologous melanoma cells. There are five types of evidence that active specific immunotherapy with the allogeneic melanoma vaccine induces an enhanced immune response directed at autologous melanoma cells:

(1) The strong correlation between the level of both humoral and cell-mediated immune response to the MCV and survival of the immunized patients suggests that the immune response so induced is cross-reactive against the metastases of autologous melanoma cells.
(2) The complete and partial regressions observed in patients with evaluable disease suggest the induction of a cross-reacting host immune response that destroys *in vivo* melanoma cells.
(3) The concomitant increase in reactivity to allogeneic and autologous melanomas by MLTR and humoral antibody response suggests the presence of shared MAA.
(4) The induction of changes in TIL-infiltrating melanoma metastases suggests the activation of lymphocyte subsets directed at MAA found on autologous melanoma cells.
(5) Our *in vitro* studies with autologous cytotoxic T cell lines clearly indicate the ability of allogeneic melanoma cells to induce sensitization to shared or cross-reactive melanoma antigens on autologous melanomas capable of rendering the cell susceptible to killing by the CTLs if there is sharing of MHC class I antigens.[52,53]

In summary, there is extensive, if not conclusive, evidence that patients treated with MCV become sensitized to their own melanoma as well as to the allogeneic melanoma, thus supporting the use of a standardized allogeneic polyvalent MCV. The long-term use of autologous melanoma vaccines is often hampered by the unavailability of sufficient autologous tumor cells for immunization or *in vitro* assays.[54] There is controversy whether autologous melanoma derived from metastases, which is the major source of specimens for active immunotherapy in metastatic disease, is sufficiently "immunogenic" for vaccine purposes.[55] Autologous metastatic melanomas, regardless of the tumor expression of MHC class II HLA-DR antigens, are generally poor stimulators of lymphocytes in MLTR.[56,57]

Our selection of irradiated whole melanoma cells for a vaccine is based on a large body of information from animal tumor–host systems, which indicates that the most effective way to induce tumor immunity in animal systems is to use the temporary growth of a living tumor cell or to immunize with viable whole irradiated tumor cells. In almost every animal tumor–host system tested, such preparations have been found to be more effective than extracts of tumor cells, soluble membrane antigens, or highly purified tumor antigens. Thus, some important aspects of the interaction between the immunogen—in this case the tumor cell and the host—appear to require the configuration of a whole cell to effectively induce antitumor immunity. For this reason, we have selected viable melanoma tumor cells grown in tissue culture but rendered incapable of prolonged growth by radiation as the immunogen in this melanoma cell vaccine. In the future, we are hopeful that we will be able to develop alternative, more immunogenic vaccines, such as anti-ids or recombinant protein MAA. Until then, the whole melanoma cell is a practical alternative.

We have selected a polyvalent MAA vaccine composed

of three different melanoma cell lines known to contain a high concentration of the six major MAAs rather than a purified antigenic preparation for the following reasons:

(1) Our present data suggest that any single antigen will be present in high concentrations in only 65% to 80% of autologous melanomas, whereas virtually all melanomas will have one or more of the six major antigens.
(2) We do not know which of the six MAAs are the most important for host rejection and protective immunity. Thus, we would have no basis for selecting one antigen over the others if we used a univalent vaccine. There also may be additional MAAs that are important in anti-tumor immunity, which we have not yet identified, but which are likely to be present in the MCV.
(3) We have demonstrated the heterogeneity in antigenic expression among melanoma biopsy specimens from different patients.[8,21,23] Furthermore, metastatic tumors may be unstable in their phenotypic and antigenic expression.[55] If the vaccine were directed at enhancing immunity to only one MAA, tumor cell clones that do not express this MAA could escape the "vaccine's" effect. This immunoselection might result in outgrowth of a "non-antigenic" clone. This would be less likely to occur if immunization were carried out with an enhanced vaccine with multiple MAAs.
(4) The question of MHC class I or II restriction has often been raised as an argument against the use of an allogeneic vaccine. This has been carefully considered and avoided by the selection of a polyvalent vaccine composed of allogeneic melanoma cells from those individual patients who share MHC class I cross-reacting antigens with >90% of melanoma patients. We have previously shown that common melanoma antigens can induce cross-reactive cytotoxic T cells by "*in vitro*" sensitization among MHC class I matched melanomas.[52,53]

Our data clearly indicate that survival of patients with AJCC stage IV metastatic melanoma, as well as AJCC stage IIIA disease, who receive our new polyvalent MCV is significantly increased compared with that of patients who receive other therapies, including our previous melanoma vaccines. Although, as shown in Table 2, historical control patients receiving other treatments showed a slightly different distribution of metastatic sites, we found that there are two reasons why the effect of MCV immunotherapy remains significant after accounting for the different distribution of first recurrent metastatic site. The first is illustrated by the stratified analysis carried out for each recurrent site group: lung, soft tissue, and liver/brain. We found the MCV group had improved survival in each site group (Figs. 12, 13, and 14), thus controlling for the effect of site.

The second line of evidence for a therapeutic effect is illustrated by the results of multivariate analysis. Careful analysis of prognostic factors by univariate and multivariate analysis indicates that differences in the mix between patients immunized with the new vaccine and control patients are not adequate to explain differences in survival. Furthermore, chronologic differences in the time intervals of treatment are not responsible for the improved survival seen in the immunotherapy patients. In fact, when all patients were analyzed by univariate and multivariate analysis, only two factors were found to be significant by multivariate analysis with regard to survival. The first site of metastasis was a significant prognostic factor, but after correcting for this and controlling for the site of metastasis, immunotherapy with the new vaccine remained a highly significant prognostic factor.

One must be very cautious in evaluating new therapies for metastatic melanoma. Metastatic melanoma is infamous for producing initial enthusiastic reports of high response rates for various chemotherapy regimens, which subsequent reports are unable to reproduce or translate into prolonged survival. Such reports, however, have usually involved small groups of patients, most of whom usually have skin and subcutaneous metastases, metastatic sites that are known to be more responsive to any type of therapy than are metastases to visceral sites. A careful analysis of our large melanoma database indicates that the natural history of melanoma, although variable in individual patients, is relatively constant when large groups are analyzed. In fact, the survival has been remarkably constant over a period of 20 years. We now report our results with this new melanoma vaccine in which we have accumulated a large enough series in a phase II trial to get a true picture of the survival of patients treated with this vaccine rather than merely report response rates that, as previous studies have shown, are not always translated into enhanced survival.

The historical experience of JWCI, against which we have compared our new vaccine, is large, and the median survivals of patients in this series equals or exceeds series reported by other investigators. Most centers have had experiences similar to that reported by Ahmann from the Mayo Clinic, who observed only ten 5-year survivors (2%) among 502 patients with advanced melanoma.[3] In our historical experience, among the patients treated by JWCI's staff, we have found a 6% 5-year survival rate, which is slightly higher than that reported by most other centers.

We have undertaken an extensive statistical review of our results in an attempt to detect a bias in favor of the group treated by immunotherapy. After such review, by every objective criteria, our results appear to be consistent that our new melanoma vaccine is responsible for significantly enhancing the survival of stage IIIA and IV mel-

anoma patients by a factor of three- or fourfold. Final confirmation of these results, however, must await validation by a properly stratified, randomized phase III trial testing immunotherapy with the new vaccine against the best alternative therapy.

It would be unrealistic to assume that we have developed an optimal melanoma vaccine at this time. Recent observations from animal tumor models and discoveries on the influence of cytokines in modulating the expression of tumor-associated antigens in various neoplasms and the host response to these antigens suggest many possible new approaches to improve melanoma vaccines.[58–69] Consequentially, we are working to improve our current melanoma vaccine in an attempt to induce a more potent immune response in a larger proportion of the immunized patients. These investigations are directed toward improving the vaccine itself, as well as investigating various biologic response modifiers with the goal of reversing immunosuppression in the patient with malignant melanoma. Our current data indicate that, among the biologic response modifiers we have investigated thus far, only cimetidine is active.[10,70]

The success we have had in achieving a high response rate with the new MCV as opposed to our prior TCV may provide a clue for preparation of other cancer vaccines. It is clear that the random selection of melanoma cell lines for our prior vaccine, without regard to their content of MAA, was unsuccessful in its ability to induce antibodies to cell surface antigens[22] and prolong survival (Fig. 15). The heterogeneity among melanomas from different patients makes it essential to quantitate MAA content and select only those cell lines that express high levels of MAA.

It is possible that our new MCV may be useful for active immunotherapy in other types of human cancer, because five of the six tumor-associated antigens found in our new vaccine (Table 1) are also present in other types of human neoplasms. The lipoprotein antigen (180 kd) is the only one whose distribution is restricted to melanoma. Although 9-0-acetylated GD3 may be restricted to melanoma, it induces cross-reacting antibodies to GD3, which is more widely distributed in other types of human neoplasms.

One of the most interesting aspects surrounding the response to active immunotherapy with the MCV observed in this study relates to the temporal relationship between the initiation of immunotherapy and objective tumor regression, which differs from that of other types of therapy. For example, the objective response to chemotherapy is usually quite rapid and often begins within a few weeks after the first course of therapy. These remissions are often of short duration and may not translate into longer survival unless they are complete. In contrast, patients receiving active immunotherapy usually do not show any evidence of disease stabilization or regression before 8 to 12 weeks, after which their disease may slowly regress over a period of 2 to 6 months. Such regressions are usually of longer duration and the patient may continue in remission for months or years. Occasionally, the response to active immunotherapy is not accompanied by obvious regression, but there is a stabilization in size of metastases or a slowing of their growth, which appears to be associated with prolongation of survival.

These observed differences in the temporal relationship between the objective responses and initiation of active immunotherapy make it inappropriate to apply to active immunotherapy the same classic criteria of response used for chemotherapy. The differences between these two types of systemic therapy for metastatic disease are understandable if one reflects on the fact that MCV immunotherapy itself has no direct effect on the metastatic neoplasm but instead must depend for its therapeutic effect on mobilization of the patient's humoral antibody and cell-mediated immunity specifically directed to attack the MAA on autologous metastases. Thus, it is not surprising that there is such a strong correlation between survival and the humoral antibody and cell-mediated immune response observed after active immunotherapy in this study. For these reasons, we believe total survival and quality of life parameters are more appropriate measurements of response to active immunotherapy than the classical criteria of response used for chemotherapy and radiotherapy.

The low toxicity of this vaccine might justify its use as the first treatment for recurrent melanoma before consideration of more toxic regimens, such as interleukin-2, lymphokine-activated killer cells, or TIL therapy[70,71] or chemotherapy,[29] whose overall long-term survival benefits have not been superior to active immunotherapy with our new polyvalent melanoma vaccine. The low toxicity of MCV also makes it reasonable to consider its use as an adjuvant in earlier stage II and III patients who are clinically free of disease after surgery.

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Discussion

DR. J. BRADLEY AUST (San Antonio, Texas): Dr. MacLean, members and guests: Dr. Morton has tilled this vineyard of immunotherapy for melanoma for a quarter of a century now, and his efforts have been characterized by enthusiasm and persistence. This is the latest in a series of papers that he has presented on this subject.

Melanoma is a chameleon of tumors; it sometimes grows very rapidly and sometimes lies dormant for many, many years, making it difficult to compare apples with apples in this field.

The concept of immunotherapy for melanoma is fostered by the fact that every now and then a nonspecific host occurrence alters the tumor growth and the tumor disappears, and sometimes it goes away with minimal therapy. This presents a difficult problem. I am not disputing at all the very nice responses that he has obtained in using this vaccine. It is clear that the patients who respond and have an effect on their tumor will do better. I just do not know how many of the patients would have responded to a nonspecific event. And that is a very difficult thing to evaluate.

If we are to be convinced that we have a vaccine that works, we cannot depend on historical controls. This is not possible in this day and age. I think that his final suggestion proposing that we need a prospective randomized double-blind multicenter study to prove the validity of this new vaccine is valid. I think we cannot substitute anything less than that to evaluate this therapy.

Dr. Morton has spent much time and energy in this field and probably knows more about immunotherapy for melanoma than anybody alive at this point. I would like to know something more about the composition of this vaccine and how difficult it is to produce and whether or not it is available in quantities to do a prospective double-blind multicenter study.

DR. DONALD L. MORTON (Closing discussion): Thank you, Dr. Aust, for bringing up the question of spontaneous regression. This frequently discussed but rarely observed phenomenon cannot explain our results. If melanoma metastasizes to distant sites, spontaneous regression is extremely rare: only one in every 1000 to 10,000 cases. Consistent tumor regression in a large number of patients simply cannot be attributed to a spontaneous event.

Why am I so confident that the vaccine has a real therapeutic effect? It is generally agreed that melanoma metastatic to distant sites is rapidly lethal. One series of 500 patients from the Mayo Clinic reported only ten 5-year survivors. In our vaccine series, however, we have 18 5-year survivors of 75 patients. This is obviously a very significant difference.

As Dr. Aust points out, the standard for evaluating any new therapy must be a multicenter, randomized phase III trial. If the National Cancer Institute approves our grant, we can produce enough vaccine to undertake such a trial within the next few years. The vaccine is composed of three melanoma cell lines specifically selected from 150 lines started and stored in our tumor-cell freezer bank over the last 20 years. We think these three cell lines are unique and important because of their high content of the six melanoma antigens defined in our laboratory that are immunogenic in humans. The vaccine is grown in tissue culture, stored in dimethylsulfoxide, and then frozen in liquid nitrogen. The only difficulty is that melanoma cells must be viable and metabolically active but unable to divide (due to irradiation). Thus the vaccine must be freshly prepared every time the patient comes for treatment, which requires the availability of a technical staff.

Still, we are anxiously looking forward to beginning the phase III trial. As Dr. Aust mentioned, we have been working on active specific immunotherapy of melanoma for 25 years. During this time, our randomized trials have found a small therapeutic effect that was not quite statistically significant. I firmly believe that the new vaccine's potential justifies initiation of a multicenter randomized trial in the very near future.